

Detection of Broadly Neutralizing Activity within the First Months of HIV-1 Infection

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ABSTRACT

A fraction of HIV-1 patients are able to generate broadly neutralizing antibodies (bNAbs) after 2 to 4 years of infection. In rare occasions such antibodies are observed close to the first year of HIV-1 infection but never within the first 6 months. In this study, we analyzed the neutralization breadth of sera from 157 antiretroviral-naïve individuals who were infected for less than 1 year. A range of neutralizing activities was observed with a previously described panel of six recombinant viruses from five different subtypes (M. Medina-Ramirez et al., *J Virol* 85:5804–5813, 2011, <http://dx.doi.org/10.1128/JVI.02482-10>). Some sera were broadly reactive, predominantly targeting envelope epitopes within the V2 glycan-dependent region. The neutralization breadth was positively associated with time postinfection ($P = 0.0001$), but contrary to what has been reported for chronic infections, no association with the viral load was observed. Notably, five individuals within the first 6 months of infection (two as early as 77 and 96 days postinfection) showed substantial cross-neutralization. This was confirmed with an extended panel of 20 Env pseudoviruses from four different subtypes (two in tier 3, 14 in tier 2, and four in tier 1). Sera from these individuals were capable of neutralizing viruses from four different subtypes with a geometric mean 50% infective dose (ID_{50}) between 100 and 800. These results indicate that induction of cross-neutralizing responses, albeit rare, is achievable even within 6 months of HIV-1 infection. These observations encourage the search for immunogens able to elicit this kind of response in preventive HIV-1 vaccine approaches.

IMPORTANCE

There are very few individuals able to mount broadly neutralizing activity (bNA) close to the first year postinfection. It is not known how early in the infection cross-neutralizing responses can be induced. In the present study, we show that bNAbs, despite being rare, can be induced much earlier than previously thought. The identification of HIV-1-infected patients with these activities within the first months of infection and characterization of these responses will help in defining new immunogen designs and neutralization targets for vaccine-mediated induction of bNAbs.

One of the main challenges for the development of a preventive human immunodeficiency virus type 1 (HIV-1) vaccine is the design of immunogens and immunization strategies that allow the induction of neutralizing antibody responses against multiple HIV-1 isolates. The main target of neutralizing antibodies is the trimeric envelope glycoprotein spike (Env). Unfortunately, when these recombinant proteins are used as immunogens, there is minimal induction of cross-reactive neutralizing antibody responses (1, 2). Despite these hurdles, high titers of broadly neutralizing antibodies (bNAbs) have been found in some chronically infected patients (3, 4). Data from many studies indicate that between 10 and 25% of patient sera displayed broadly neutralizing capacity against diverse HIV-1 strains (2–5), and one large study showed that 50% of sera from chronic infection can neutralize 50% of virus strains (6). Antibody responses have been studied extensively in these chronically infected patients, new bNAbs have been isolated, and the molecular determinants recognized by these antibodies have been characterized. Although these antibodies do not protect infected patients, they exert selective pressure on the virus (7–9). On the other hand, and more importantly, passive transfer of broadly neutralizing antibodies to monkeys and humanized mice effectively protects them against chimeric simian-

human immunodeficiency virus (SHIV) infection and HIV infection, respectively (10–18). Additionally, promising results have been obtained in recent years with approaches based on the production of broadly neutralizing antibodies against HIV and SHIV, or molecules resembling these antibodies, by adeno-associated vectors in animal models (19, 20). In humans, passive transfer of bNAbs also has shown some efficacy in controlling viral replication in two recent studies, showing that passive transfer of mono-

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clonal antibody targeting the CD4 binding site can reduce HIV-1 viremia (21, 22).

Taking into account all of the above-described findings, it is reasonable to think that the knowledge of the mechanisms involved in the development of this type of antibody will provide valuable information for the design of an efficient HIV vaccine. Several studies of chronically infected patients have found a positive correlation between viral load and neutralization breadth (5, 23, 24). Broadly cross-reactive neutralizing activity also has been associated with partial B cell restoration (24). In addition, it has been proposed that long periods of viral replication were required to induce the high levels of somatic mutation found in the vast majority of bNAbs (25–27). These data suggest that the design of immunogens and immunization strategies that produce bNAbs will be complicated. To help guide such strategies, the identification and characterization of broadly neutralizing responses in recent infection would provide valuable information.

Upon infection with HIV-1, essentially all individuals develop a strong antibody response against the viral Env. Within 1 week of detectable viremia (10 to 12 days after infection), antigen-antibody complexes are detected. Following this phase, antibodies directed against gp41 and gp120 and to conserved regions (CD4-binding site [CD4bs] and membrane-proximal external region [MPER]) are generated after 23, 38, and 40 to 70 days postinfection, respectively. Unfortunately, despite these antibodies exerting selective pressure on the virus, there is no effect on viremia levels (28).

There is some evidence that the induction of broadly neutralizing antibodies during the first year of infection is possible, although it is less frequent than that in chronic infection. Two recent reports described three patients that developed broadly neutralizing activity (bNA), defined as the ability of serum to neutralize viruses from different clades with a geometric mean 50% infective concentration (IC_{50}) between 100 and 500, at 9.8 and 12 months postseroconversion (29, 30).

In order to better understand bNA induction during recent infection, in the present study we have studied neutralization breadth in patients within the first year of HIV-1 infection. In addition, we have explored the possibility of finding these responses as early in HIV-1 infection as possible. In order to do so, we have studied the neutralization breadth in a cohort of 157 antiretroviral-naïve recently infected individuals (<1 year postinfection) and compared the results obtained with a similar analysis done in a cohort of 170 untreated chronically infected individuals (>1 year postinfection).

MATERIALS AND METHODS

Study participants. Samples from 327 HIV-1-infected individuals treated at the Hospital Clinic of Barcelona (Spain) were collected, 157 within less than 365 days postinfection (dpi) and 170 after more than 365 dpi. A cross-sectional study with samples of these patients was carried out. All individuals gave informed written consent, and the study was reviewed and approved by the Institutional Review Board (IRB) of the Hospital Clinic of Barcelona (Spain).

Recent infection testing algorithm. In order to detect the presence of HIV-specific antibodies, a conventional sensitive enzyme immunoassay (AxSYM system based; Abbott Laboratories, North Chicago, IL, USA) or Western blotting (Inno-LIA HIV I/II score; Innogenetics, Ghent, Belgium) was used. For the detection of plasma HIV-1, an RNA Cobas Amplicor monitor was used (Roche Molecular Systems, Branchburg, NJ, USA). For the identification of recent infections, the test for recent infec-

tion (t.r.i.) used was BED-CEIA (modified version of the Vironostika HIV-1-1 enzyme immunoassay; bioMérieux Durham, NC, USA) (31).

For the <365-dpi group of patients, we designated a specific recent infection testing algorithm to identify cases of recent infection and to divide them into three subgroups depending on the days postinfection: <100 (early recent), 100 to 180 (late recent), and 180 to 365 (early chronic) dpi. The detailed flow chart used for dating HIV infection based on Western blot results, the test for recent infection, and days since previous negative serology is shown in Fig. 1. The characteristics of the cohort of patients with early recent and late recent HIV infection, belonging to the acute/recent HIV infection cohort at the hospital clinic, are described elsewhere (32).

For the group of chronically infected patients we selected 170 serum samples from different individuals known to have tested positive by ELISA and viral load at least 1 year prior to the sample.

Neutralization assays. Serum samples were tested in triplicate at a 1/200 dilution with the six recombinant viruses representing 5 different genetic subtypes using TZM-bl target cells as previously described (33). The minipanel of replication-competent recombinant viruses was previously generated by replacing the *env* sequence of HIV NL4-3 with *env* sequences from isolates of 5 different subtypes: VI 191, 92BR025, 92UG024, CM244, and AC10.0.29 (33). An amphotropic vesicular stomatitis virus (VSV) Env pseudotyped on an HIV-1 core was added to the panel as a specificity control virus in neutralization assays. Pseudotyped viruses for neutralization assays were generated by manual or automated large-scale production of HIV by cotransfection of an HIV-1 *env* expression plasmid and an *env*-deficient HIV-1 backbone (pSG3ΔEnv), as previously described (34, 35). Clades, tropisms, and tier categorizations are indicated in Fig. 4. Viruses have been classified into tiers according to their sensitivity to neutralization: very high (tier 1A), above average (tier 1B), moderate (tier 2), and low (tier 3) sensitivity (36). The Fiebig classification (I to VI) in different stages of primary infection is based on the emergence of viral markers (37).

Twenty-five-microliter aliquots of the corresponding serum 2-fold dilution (1/100 to 1/6,400) in DMEM–10% fetal bovine serum was added. All serum samples were heat inactivated at 56°C for 30 min before use in neutralization assays. Each virus in a total volume of 75 μ l then was added to the corresponding wells. The amount of each virus chosen was the lowest level of viral input sufficient to give a clear luciferase signal within the linear range for each virus. The plate was incubated for 1 h at 37°C. After incubation, 10^4 target cells (TZM-bl) in a volume of 100 μ l were added to each well. The plate then was placed in a humidified chamber within a CO₂ incubator at 37°C. Luciferase activity in cell lysates was assessed 72 h after infection for recombinant viruses and 48 h after infection for pseudotyped viruses. Luciferase activities in cell lysate infected with nonneutralized viruses were considered 100%. We defined cross-reactive neutralizing responses as the neutralization of at least one virus included in the previously described minipanel from at least three different subtypes and bNA as the ability of serum to neutralize viruses from different clades with a geometric mean 50% infective dose (ID_{50}) titer between 100 and 500 as previously described (29, 30).

Epitope mapping. (i) Neutralization assays. For the characterization of neutralizing antibodies, neutralization assays with single-round HIV-1 Env pseudovirus infection were performed using a luciferase-based assay in TZM-bl cells (38). To determine the serum concentration causing a 50% reduction of relative light unit value, 4-fold dilutions (1/20 to 1/327,680) were made. The neutralization dose-response curves were fitted by nonlinear regression using a four-parameter hill slope equation. For the assessment of CD4 binding site (CD4bs)-directed neutralization, serum neutralization was blocked with specific protein probes (RSC3 or RSC3 3711/P363N) in a competition assay as previously described (39). For the mapping of neutralizing antibodies directed to glycan structures in the variable region (V1V2 and V3), neutralization assays were performed using JRCSF virus with the N160K mutation and the N332A mutation, respectively (3, 40). For the mapping of NAbs to the MPER of gp41,

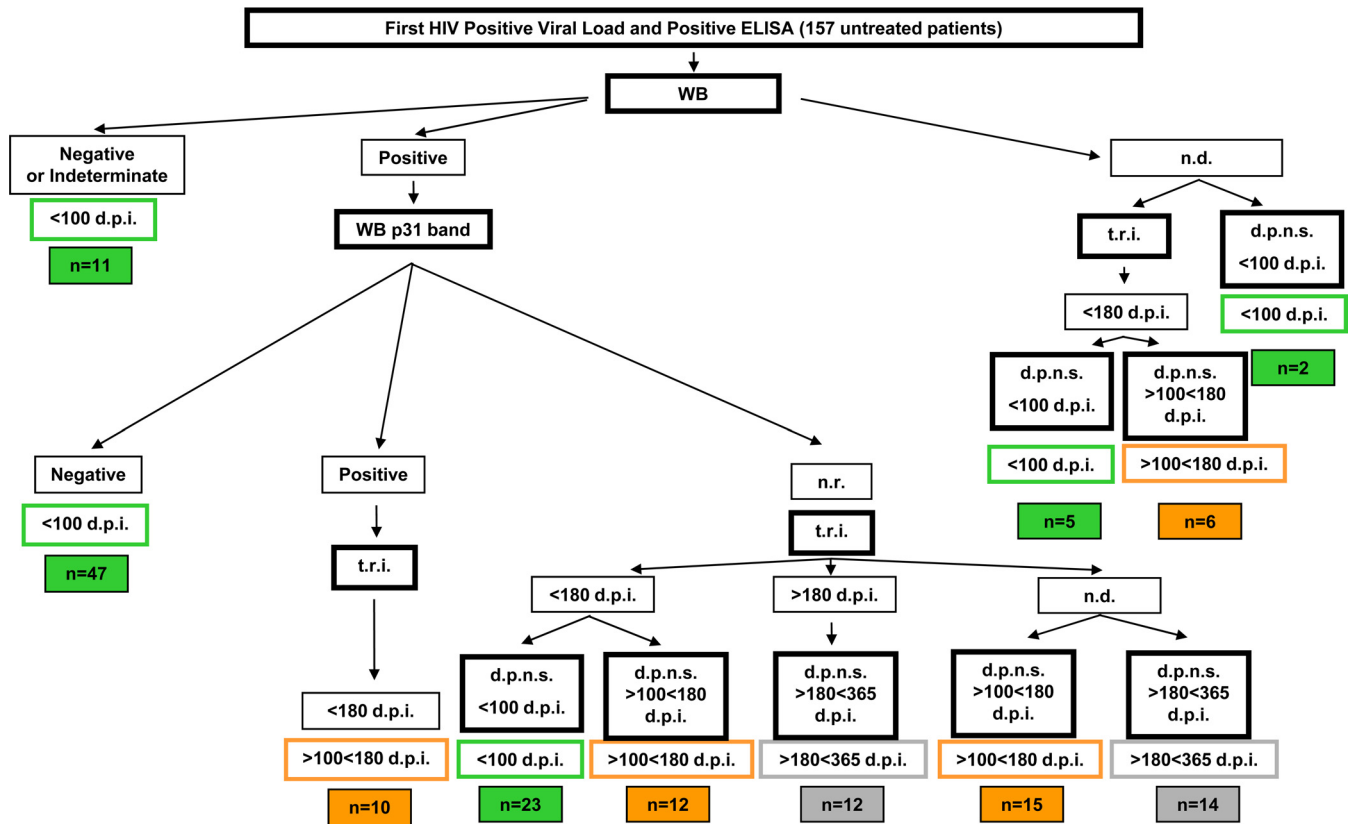


FIG 1 Algorithm for dating of HIV-1 infection in patient sera (<100 dpi, >100 but <180 dpi, and >180 but <365 dpi). d.p.n.s., days postnegative serology; t.r.i., test for recent infection; n.d., not done; n.r., not reported. The following categories were used for samples from patients infected for <100 dpi: (i) negative or indeterminate Western blot (WB); (ii) positive Western blot with a negative p31 band; (iii) positive Western blot with no data for the p31 band and t.r.i. indicating fewer than 180 days of infection, and d.p.n.s. indicating fewer than 100 days of infection; (iv) no Western blot information, with t.r.i. indicating less than 180 days of infection and d.p.n.s. indicating less than 100 days of infection; and (v) d.p.n.s. within 100 days prior to sampling. The following categories were used for samples from patients infected for 100 to 180 dpi: (i) positive Western blot with a positive p31 band and a t.r.i. indicating less than 180 days of infection; (ii) positive Western blot with no data for the p31 band, a t.r.i. indicating less than 180 days of infection, and a d.p.n.s. indicating 100 to 180 days prior to the sampling; (iii) positive Western blot with no p31 band data reported, no t.r.i. data, and a d.p.n.s. of within 100 to 180 days prior to the sampling; and (iv) no Western blot information, t.r.i. indicating less than 180 days of infection, and a d.p.n.s. within 100 to 180 days prior to the sampling. The following categories were used for samples of patients infected for 180 to 365 dpi: (i) positive Western blot with no data for the p31 band, t.r.i. indicating more than 180 days of infection, and a documented previous negative serology within 180 to 365 days prior to sampling; (ii) a positive Western blot with no data for the p31 band, no t.r.i. data, and a d.p.n.s. of within 180 to 365 days prior to sampling.

sera were tested for neutralizing activity against a chimeric HIV-2 virus containing the MPER of HIV-1 (71312.C1) and the parental HIV-2 7312A clone (41, 42). The serum neutralizing antibodies also were mapped to the MPER region by a method of selective peptide inhibition of neutralization (43).

(ii) **ELISAs.** Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (39, 44). The ELISA plates were coated with the following probes: HxB2 gp120wt and HxB2 gp120 D368R proteins, the antigenically resurfaced glycoprotein RSC3 containing the CD4bs, the RSC3 P363N mutant, and the RSC3 G367R probe. Sera that showed a loss of reactivity to the CD4bs mutants (HxB2 D368R and RSC3 P363N) and reacted to both RSC3 and RSC3 G367R were classified as containing CD4bs antibodies.

Statistics. Analyses were performed using GraphPad Prism 5 (GraphPad Software). Mann-Whitney U test was used for comparisons of continuous variables between groups. Simple comparisons were made with the use of a two-sided alpha level of 0.05.

RESULTS

Demographic characteristics. In the <365-dpi group, 95.5% of participants were male and 4.5% were female. Heterosexual trans-

mission was reported for 8.9% of the patients, while men who have sex with men (MSM) accounted for 86.6% of the study population. Intravenous drug use (IDU) accounted for 1.3% of the study population. In the >365-dpi group, 77.6% of participants were male and 22.4% were female. Heterosexual transmission was reported for 24.7% of the patients, while men who have sex with men accounted for 65.9% of the study population. Intravenous drug use accounted for 7.1% of the study population (Table 1). In the <365-dpi group, all patients were treatment naive. The chronically infected individuals (>365 dpi) with a history of treatment at the time of sampling had been off treatment for at least 3 years. Clinical characteristics of the patients are shown in Table 1. The median and range for each patient group for CD4⁺ T cell count, viral load (VL), and number of years since HIV diagnosis at the time of screening are shown.

Identification and characterization of patients infected with HIV for less than 1 year. We studied neutralization breadth in a group of 157 patients with recent HIV-1 infection. Within these patients, 88 were infected for less than 100 days (termed early

TABLE 1 Clinical characteristics of patient groups

Parameter	Value(s) for group:		P value ^d
	<365 dpi (n = 157)	>365 dpi (n = 170)	
Gender (no. [%])			
Male	150 (95.5)	132 (77.6)	<0.001
Female	7 (4.5)	38 (22.4)	
Age (median yr [IQR])	34 (28, 39)	41 (34, 46)	
Origin (no. [%])			0.065
Western Europe	106 (67.5)	130 (76.5)	
Eastern Europe	1 (0.6)		
South America	37 (23.6)	22 (12.9)	
Caribbean	3 (1.9)	6 (3.5)	
North America	4 (2.5)	1 (0.6)	
Sub-Saharan Africa		1 (0.6)	
Northern Africa	1 (0.6)		
Other	5 (3.2)	10 (1.7)	
Transmission route (no. [%])			0.003
MSM	136 (86.6)	112 (65.9)	
Heterosexual	14 (8.9)	42 (24.7)	
Bisexual	5 (3.2)	2 (1.2)	
IDU	2 (1.3)	12 (7.1)	
Time since infection ^a (median days [IQR])	94 (52, 137)	1,962 (887, 3,607)	<0.001
Early recent (n = 88)	56 (37, 73)		
Late recent (n = 43)	131 (111, 145)		
Early chronic (n = 26)	203 (182, 222)		
Symptomatic primary infection (no. [%])	89 (56.7)		
Fiebig stage ^b (no. [%])			
I			
II	2 (1.3)		
III	4 (2.5)		
IV	6 (3.8)		
V	75 (47.8)		
VI	70 (44.6)	170 (100)	
Subtype ^c (no. [%])			0.086
B	125 (79.6)	57 (33.5)	
Non-B	21 (13.4)	4 (2.4)	
Unknown	11 (7)	109 (64.1)	
CD4 ^b (median no. of cells/ μ l [IQR])	479 (366, 629.5)	559.50 (435.75, 753.75)	0.489
CD8 ^b (median no. of cells/ μ l [IQR])	905 (605.3, 1,415.3)	1094 (859.75, 1,591.5)	0.041
CD4 ⁺ /CD8 ⁺ (median no. of cells/ μ l [IQR])	0.4 (0.19, 2.28)	0.5 (0.36, 0.71)	<0.001
Plasma VL ^b (median no. of copies [IQR])	55,466 (16,652, 178,436)	8,898 (2,427, 24,310)	<0.001
Plasma VL log ₁₀ ^b (median [IQR])	4.73 (4.22, 5.27)	3.94 (3.38, 4.38)	<0.001

^a Estimated days of infection.

^b At the time of sample collection.

^c According to protease and retrotranscriptase sequences.

^d Mann-Whitney U test was used for continuous variables, and for simple comparisons a two-sided alpha level of 0.05 was used.

recent infection), 43 were infected for more than 100 days but less than 180 days (termed late recent infection), and 26 patients were infected for more than 180 days but less than 365 days (termed early chronic infection). Regarding Fiebig stages of infection (37), the majority of patients were classified as stages V and VI (47.8% and 44.6%, respectively), and a small proportion were recently infected and classified as stages II, III, and IV (1.3%, 2.5%, and 3.8%, respectively) (Table 1).

We next calculated the estimated day of infection for all samples from the <365-dpi group as 20 days before the symptom onset date for symptomatic subjects or the midpoint between the last negative and first positive HIV test for those lacking symptoms (28, 45). The median estimated duration of infection for the patients with less than 1 year of infection was 94 days (interquartile range [IQR], 52 to 138). Furthermore, according to the three groups established for patients infected for less than 1 year, the

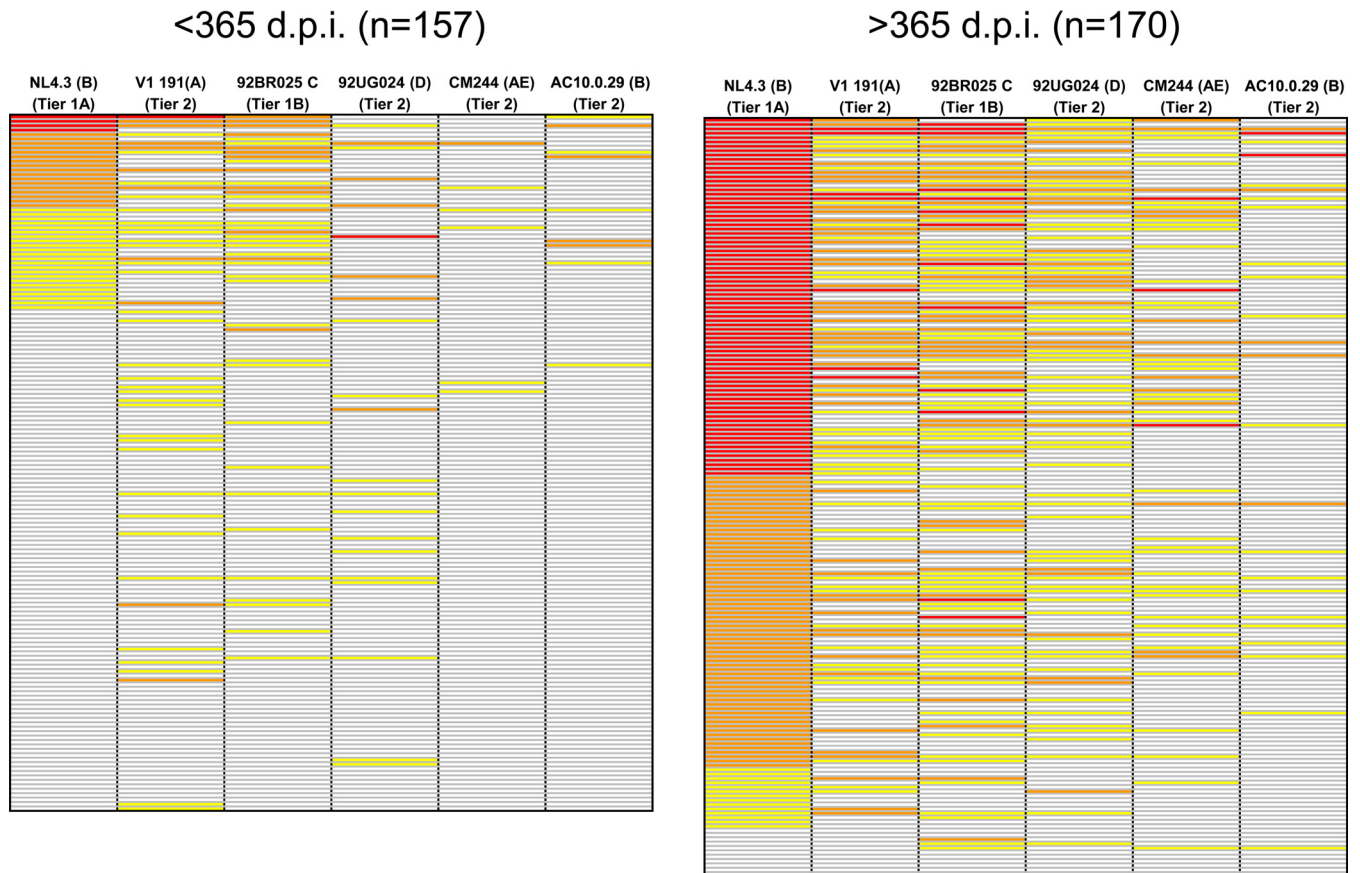


FIG 2 Neutralization data for patients infected for less than 365 days and patients infected more than 365 days against the minipanel of viruses. Shown are percentages of neutralization at a 1/200 serum dilution. A white box indicates <50% neutralization, a yellow box indicates $\geq 50\%$ and <70% neutralization, an orange box indicates $\geq 70\%$ and <90%, and a red box indicates $\geq 90\%$ neutralization.

median estimated duration of the infection in early recent infection was 56 days (IQR, 37 to 73); the median estimated duration for patients with late recent infection was 131 days (IQR, 111 to 145), and the median estimated duration of infection for early chronic patients was 203 days (IQR, 184 to 222) (Table 1).

As a control group, we selected 170 individuals with evidence of chronic HIV-1 infection (more than 365 dpi since the first HIV-positive sample) from a previous cross-sectional study (33). At the time of sampling, these samples had a median estimated duration of infection, calculated as the time since the first positive HIV serology to the time of sampling, of 1,962 days, or 5.4 years (IQR, 887 to 3,607 days) (Table 1).

Initial screen for cross-neutralizing sera. We next sought to use the screening panel to survey all of the serum samples (157 from the <365-dpi group and 170 from the >365-dpi group) for cross-neutralizing activity. All of the samples were tested against a previously described 6-virus minipanel (Fig. 2). This minipanel includes 6 viruses from 5 different subtypes (33). Each virus-serum sample combination was analyzed in triplicate at 1/200 dilution. Figure 2 summarizes the neutralization results for the 331 serum samples.

Detectable cross-neutralizing responses, defined as neutralization of at least one virus from at least three different subtypes, were observed in sera from 21 patients infected for less than 1 year. The selection criterion for identification of cross-neutralizing re-

sponses used in the present study was less restrictive than that of previous studies done with the same virus panel due to the relevance and rarity of these responses (24, 33). Significant differences in neutralization breadth were observed between both groups (<365 dpi and >365 dpi) (Fig. 3A). A total of 13.4% (21 out of 157) of the samples from <365-dpi patients achieved 50% neutralization of at least one recombinant virus from three different subtypes at a $\geq 1/200$ dilution. In contrast, the percentage of samples from >365-dpi patients capable of neutralizing at least one recombinant virus from three different subtypes was 58.8% (100 out of 170). No significant neutralization of the VSV pseudotyped control was observed in any of the samples analyzed. It is important to note that, despite being much less frequent ($P = 0.001$), we have detected cross-neutralizing responses in patients infected for less than 1 year. Remarkably, 3.8% of the samples from <365-dpi patients were capable of neutralizing viruses from at least 4 different subtypes (6 out of 157) (Fig. 3A).

We then analyzed differences in neutralization breadth among the three groups of patients infected for less than 1 year (early recent, late recent, and early chronic infection) (Fig. 3B). The percentage of patients capable of neutralizing at least one virus from three different subtypes was 5.7% (5 out of 88) in early recent infection (<100 dpi), 13.9% (6 out of 43) in late recent infection (100 to 180 dpi), and 38.5% (10 out of 26) in early chronic infection (180 to 365 dpi). These results agree with previous reports

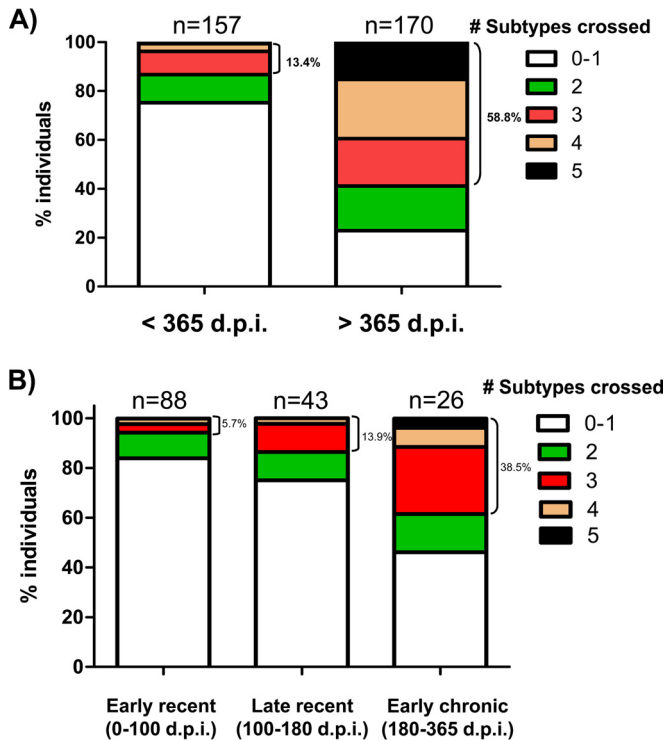


FIG 3 Differences in neutralization breadth in patients from different times postinfection. The percentages of patients capable of neutralizing at least one virus from different subtypes out of a previously described minipanel in each patient group are shown. The number of subtypes crossed are indicated with different colors. The numbers of patients per group are indicated. The percentages of patients capable of neutralizing at least one virus from 3 different subtypes also are indicated. (A) Comparison between <365-dpi and >365-dpi patient groups. (B) Comparison among three patient groups within the first year of infection (0 to 100, 100 to 180, and 180 to 365 dpi).

that showed an increase in the frequency of broadly neutralizing responses over time in chronically infected patients, but to our knowledge, this is the first time that this effect has been described in patients infected with HIV for less than 1 year (28, 46). It is important to note that 11 of the 21 patients (52.3%) that showed a significant neutralization breadth within the first year of infection (5 in early recent and 6 in late recent infection) showed detectable broad cross-neutralizing responses within the first 6 months of infection.

Confirmation of neutralization breadth in patients that showed cross-neutralizing responses within the first 6 months of infection. Due to the potential interest of bNA responses induced so early after seroconversion, we next further characterized the cross-neutralizing activities detected within the first 6 months of infection against both the minipanel and an extended virus panel by making complete neutralization curves and by calculating the ID₅₀ values. In this study, we have used the definition of bNA previously used by van den Kerkhof et al. (“the ability of serum to neutralize viruses from different clades with a geometric mean IC₅₀ titer between 100 and 500”) (30).

From the available sera from patients within the first 6 months of infection, we selected 2 patients infected for <100 dpi (DET 887 and DET 909) and 3 patients infected for >100 but <180 dpi (DET 002, DET 802, and DET 1505) capable of neutralizing at least one virus from three different subtypes from the minipanel of recombinant viruses. Clinical characteristics of the selected patients are shown in Table 2. The other 6 samples with cross-neutralizing activity within the first 6 months of infection could not be further characterized for sample availability reasons. The selected samples also were tested against a new panel with 20 additional pseudotyped viruses from 4 different subtypes and recombinant forms. In order to increase the stringency of our analysis, we chose preferably difficult-to-neutralize viruses (2 tier 3, 14 tier 2, and 4 tier 1). Complete neutralization curves were done with these sera and viruses from both panels (6-recombinant-virus minipanel

TABLE 2 Clinical characteristics of 5 of the patients that showed a significant cross-neutralizing response in the first 6 months of infection

Parameter	Value for ^a :				
	DET 887	DET 909	DET 002	DET 802	DET 1505
Time since infection (days)	96	77	174	180	119
Infection subgroup	Early recent	Early recent	Late recent	Late recent	Late recent
% viruses neutralized (extended panel)	34.6	15.3	19.2	34.6	26.9
No. of subtypes neutralized	6	3	4	4	4
Gender	Male	Male	Male	Male	Male
Age (yr)	42	26	43	31	26
Origin	Western Europe	Western Europe	Western Europe	Western Europe	Western Europe
Transmission route	MSM	MSM	IDU	Bisexual	Heterosexual
Test for recent infection (<180 days)	Positive	Positive	Positive	Positive	ND
Western blot (p31 band) result	Negative	Negative	ND	ND	ND
Symptomatic infection	Yes	Yes	No	No	No
Feibig stage	V	V	VI	VI	VI
Subtype (retrotranscriptase/protease)	B/B	B/B	B/CRF02AG	B/B	B/B
Viral tropism	Dual	R5	ND	R5	R5
CD4 (cells/μl)	653	234	536	780	216
CD8 (cells/μl)	658	1,458	2,642	ND	445
CD4 ⁺ /CD8 ⁺	0.99	0.16	0.20	ND	0.49
VL (copies/ml)	76,311	111,008	148,000	5,271	24,630
Log ₁₀ VL	4.88	5.05	5.17	3.72	4.39

^a ND, not determined.

Virus	Type	Subtype/CRF	Tier	State of infection	Patients				
					DET 887	DET 909	DET 002	DET 802	DET 1505
VI 191	RV	A	2	Unknown	<100	300	200	130	100
CM244	RV	AE	2	Fiebig VI	340	<100	<100	<100	<100
NL4-3	RV	B	1A	Fiebig VI	200	>800	250	350	450
AC10.0.29	RV	B	2	Fiebig III	320	<100	100	150	400
92BR025	RV	C	1B	Fiebig VI	220	200	100	500	400
92UG024	RV	D	2	NA	210	<100	100	120	100
HIV-Q842.d12	PSV	A1	2	Acute/Early	<100	<100	n.d.	<100	n.d.
HIV-Q23.17	PSV	A1	1B	Fiebig VI	<100	<100	n.d.	<100	<100
HIV-Q769.d22	PSV	A1	2	Acute/Early	150	<100	n.d.	<100	<100
HIV-Q259.d2.17	PSV	A1	2	Acute/Early	<100	<100	n.d.	<100	<100
HIV-TRO.11	PSV	B	2	Fiebig III	110	<100	<100	<100	n.d.
HIV-QH0692.42	PSV	B	2	Fiebig V	<100	<100	<100	<100	<100
HIV-PVO.4	PSV	B	3	Fiebig III	<100	<100	<100	<100	<100
HIV-Bx08.16	PSV	B	1B	Fiebig VI	<100	160	n.d.	250	150
HIV-RHPA4259.7	PSV	B	2	Fiebig \leq V	<100	<100	n.d.	<100	<100
HIV-SC422661.8	PSV	B	2	Fiebig IV	<100	<100	n.d.	<100	n.d.
HIV-TRJO4551.58	PSV	B	3	Fiebig II	<100	<100	n.d.	<100	<100
HIV-THRO4156.18	PSV	B	2	Fiebig II	<100	<100	n.d.	<100	<100
HIV-CH117.4	PSV	BC	2	Fiebig VI	<100	<100	n.d.	<100	<100
HIV-CH181.12	PSV	BC	2	Fiebig VI	200	<100	<100	<100	n.d.
CHI-CH110.2	PSV	BC	2	Fiebig VI	<100	<100	n.d.	<100	<100
HIV-CAP45.2.00.G3	PSV	C	2	Fiebig IV	<100	<100	n.d.	190	n.d.
HIV-Du422.1	PSV	C	2	Fiebig V	<100	<100	<100	100	n.d.
HIV-16055 2.3	PSV	C	2	Fiebig II	<100	<100	n.d.	<100	n.d.
HIV-TV1.21	PSV	C	1B	Fiebig VI	<100	<100	n.d.	<100	125
HIV-25711 2.4	PSV	C	1B	Fiebig III	150	<100	n.d.	250	<100

FIG 4 Serum neutralization (ID_{50} s) data from 5 patients with a broad neutralization profile within the first 6 months of infection. Numbers indicate ID_{50} s. An orange box indicates an ID_{50} of ≥ 250 , a yellow box indicates an ID_{50} of ≥ 100 but < 250 , and a white box indicates an ID_{50} of < 100 . RV, recombinant virus; PSV, pseudotyped virus; NA, not available; n.d., not done.

and 20-pseudovirus panel). In total, 5 selected serum samples were tested against 26 viruses from 6 different subtypes and recombinant forms, different states of infection (Fiebig classification [34]), different tropisms, and different sensitivities to neutralization (tier classification [33]). The characteristics of each virus are shown in Fig. 4. Reciprocal dilutions of sera that reduce infectivity by 50% (ID_{50} s) calculated from these curves also are depicted in Fig. 4. This new panel confirmed that the neutralizing activity of the analyzed viruses (previously selected with the 6-virus panel) was broadly reactive.

Impact of plasma viral load levels on neutralization breadth in the first year of infection. In order to investigate in our patients the previously reported correlation between neutralization breadth and plasma viral load levels, we evaluated the levels of viremia in patients with cross-neutralizing responses and patients with less neutralization breadth. Data on all patients, infected for more or less than 1 year, were compiled and analyzed by comparing samples from patients capable of neutralizing viruses across 3 or more subtypes (cross-neutralizing response) and samples from patients that neutralized viruses from fewer than 3 subtypes. Our analysis revealed that the levels of viremia were significantly higher for patients infected for more than 1 year and showing cross-neutralizing responses ($P = 0.0309$) (Fig. 5A). However, no differences were observed when we compared the levels of viremia between patients capable of neutralizing three or more subtypes and patients that neutralized fewer than three subtypes within the first year of infection (Fig. 5A).

Impact of the time post-HIV-1 infection on neutralization breadth within the first year of infection. We next investigated the potential relationship between neutralization breadth and time postinfection in our patients. A univariate model analysis showed that, in patients infected for more than 1 year, the number

of days postinfection were similar in patients with broad neutralization and patients with less neutralization breadth (Fig. 5B). In contrast, the number of days postinfection was significantly higher for patients with cross-neutralizing responses and less than 1 year of infection ($P = 0.0001$). Our results showed that there is a strong correlation between neutralization breadth and time postinfection during the first year of HIV infection. However, this correlation was not observed when patients were infected for more than 1 year (Fig. 5B).

Impact of $CD4^+/CD8^+$ T lymphocyte ratios and the presence of symptomatic primary infection on neutralization breadth within the first year of infection. We also investigated the potential association of neutralization breadth with the $CD4^+/CD8^+$ ratio. It has been suggested that the cutoff ratio of < 1.0 is associated with immunosenescence and mortality (47–49). We therefore evaluated the levels of neutralization breadth (number of subtypes crossed) in patients with $CD4^+/CD8^+$ ratios of < 1 and ≥ 1 . Data on all patients from < 365 - and ≥ 365 -dpi groups were compiled and analyzed by comparing samples from patients with $CD4^+/CD8^+$ ratios of ≥ 1 and patients with $CD4^+/CD8^+$ ratios of < 1 . Our analysis revealed that neutralization breadth was significantly higher in patients with a healthy $CD4^+/CD8^+$ ratio (≥ 1) within the first year of infection ($P = 0.0328$) (Fig. 6A). In addition, the breadth of the humoral immune response in patients with an asymptomatic primary infection is also significantly higher ($P = 0.0002$) (Fig. 6B). Both the absence of symptoms during primary infection and the maintenance of $CD4^+/CD8^+$ ratios above 1 can be interpreted as a consequence of a lower level of immune damage during recent infection. In fact, in the present study we have observed an asymptomatic primary infection is significantly associated with higher $CD4^+/CD8^+$ ratios during the first year of HIV

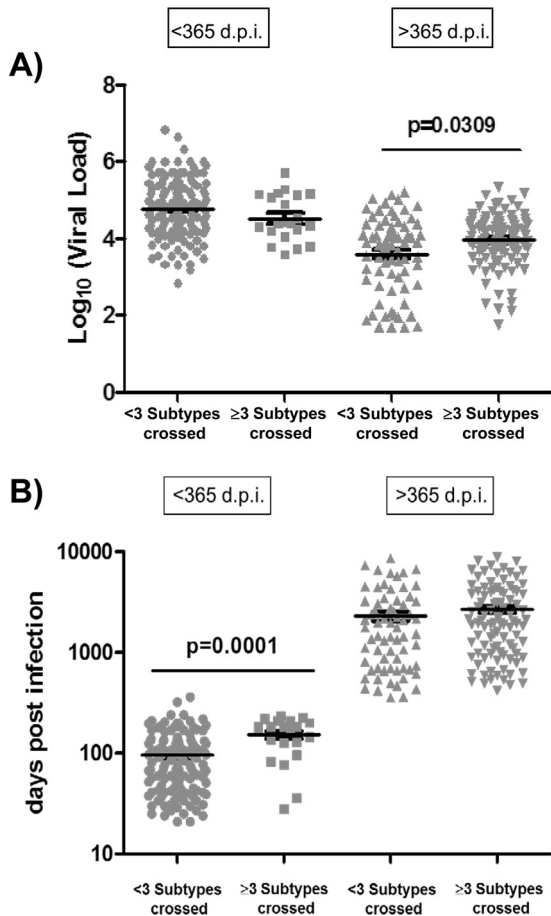


FIG 5 Viral loads and days postinfection in patients with different neutralization breadths (<3 subtypes crossed and ≥ 3 subtypes crossed). Statistical analysis of the viral load levels (A) and the days postinfection (B) is shown. Horizontal bars within the point plots indicate the median for each group \pm standard errors of the means (SEM). Significance between groups is indicated above groups. Each symbol indicated a different group of patients. Gray circles indicate <3 subtypes crossed within the <365-dpi group, gray squares indicate ≥ 3 subtypes crossed within the <365-dpi group, gray triangles indicate <3 subtypes crossed within the >365-dpi group, and gray inverted triangles indicate ≥ 3 subtypes crossed within the >365-dpi group. Mann-Whitney U tests were used for comparisons between groups. Simple comparisons were made with the use of a two-sided alpha level of 0.05.

infection ($P = 0.0002$) (Fig. 6C). Contrary to what was observed during the first year of infection, we found no differences in neutralization breadth between patients with $CD4^+/CD8^+$ ratios of ≥ 1 or < 1 in chronic infection (Fig. 6A).

We also compared some additional clinical and demographic data for all patients (<365-dpi group and >365-dpi group) to the presence of cross-neutralizing activity and observed no association with gender, age, origin, transmission route, or subtype (data not shown). We cannot rule out that some of the clinical or demographic variables analyzed are associated with the presence of cross-neutralizing activity, but we have not been able to establish these associations with the group of patients included in our study.

Epitope mapping. The epitopes targeted by the neutralizing antibodies present in sera from 4 patients with recent infection (<180 dpi) and 6 early chronic patients (180 to 365 dpi) with

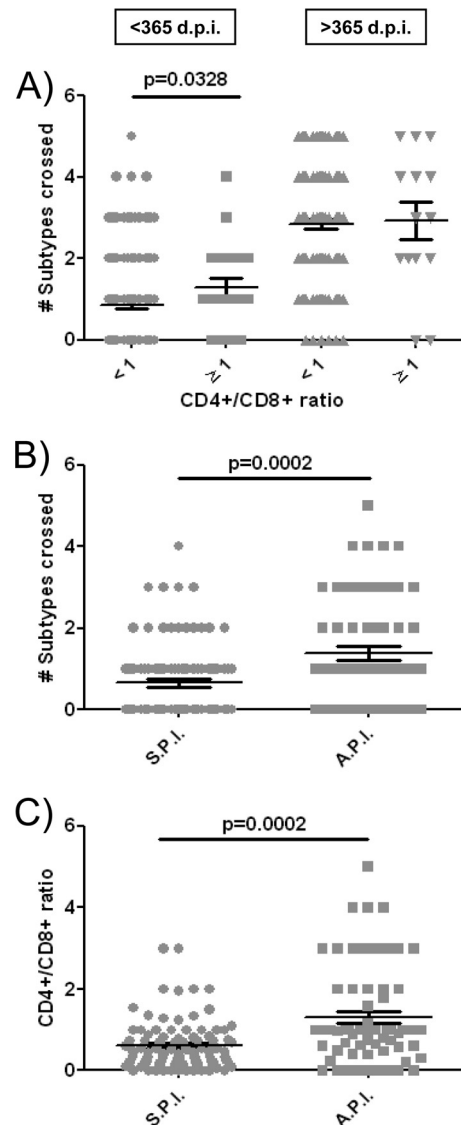


FIG 6 Neutralization breadths in patients with different $CD4^+/CD8^+$ ratios and, within the first year of infection, with or without symptomatic primary infection. (A) Statistical analysis of $CD4^+/CD8^+$ ratios, (B) the presence or absence of symptomatic primary infection in the <365-dpi group, and (C) $CD4^+/CD8^+$ ratios in patients with or without primary infection within the <365-dpi group. Horizontal bars within the point plots indicate the medians for each group \pm SEM. Significance between groups is indicated above groups. Each symbol indicated a different group of patients. (A) Gray circles indicate $CD4^+/CD8^+$ ratios of < 1 and <365 dpi, gray squares indicate $CD4^+/CD8^+$ ratios of ≥ 1 and <365 dpi, gray triangles indicate $CD4^+/CD8^+$ ratios of < 1 and >365 dpi, and gray inverted triangles indicate $CD4^+/CD8^+$ ratios of ≥ 1 and >365 dpi. (B and C) Gray circles indicate symptomatic primary infection (S.P.I.), and gray squares indicate asymptomatic primary infection (A.P.I.). Mann-Whitney U tests were used for comparison between groups. Simple comparisons were made with the use of a two-sided alpha level of 0.05.

cross-neutralizing profiles (capable of neutralizing at least one virus from 3 subtypes from the minipanel) were mapped (Fig. 7 to 10). The remaining 11 sera with cross-neutralizing profiles were not further characterized for sample availability reasons. The presence of antibodies directed against CD4bs, glycans in the V2 loop region, glycans in the V3 loop, and

	Serum	CD4bs	V3 Glycan dependent	V2 Glycan dependent	MPER	Serum description
<180 d.p.i.	DET 909	-	-	++	-	V2 glycan
	DET 887	-	-	++	-	V2 glycan
	DET 002	+	-	++	-	CD4bs, V2 glycan
	DET 1505	+	-	-	-	CD4bs
	DET 802	n.d.	n.d.	n.d.	n.d.	n.d.
>180-365 d.p.i.	DET 885	-	-	++	-	V2 glycan
	DET 727	-	-	+	-	V2 glycan
	DET 1363	+	-	++	-	CD4bs, V2 glycan
	DET 943	-	+	-	+++	V3 glycan, MPER
	DET 1427	+	-	-	-	CD4bs
>365 d.p.i.	DET 837	+	-	+	++	CD4bs, V2 glycan, MPER
	734-000	-	+	-	-	V3 glycan
	600-003	-	+++	-	+++	V3 glycan, MPER
	521-006	+	-	-	+	CD4bs, MPER
	363-014	+	+	-	-	V3 glycan
	308-40	+	-	-	+	CD4bs, MPER
	528-006	-	-	-	-	-
	642-007	+	+	-	+++	CD4bs, V3 glycan, MPER

FIG 7 Summary of the serum specificities of the sera from patients with a broad neutralization profile in recent (less than 180 dpi), early chronic (180 to 365 dpi), and chronic (>365 dpi) infection. The presence of antibodies against the four epitopes (CD4bs, V3 glycans, V2 glycans, and MPER) is represented by plus signs as follows. For CD4bs, + indicates RSC3 protein competition with <30% reduction in ID₅₀ but ELISA differences in binding RSC3/DRSC3 and/or differences in binding G367R/DRSC3. Binding was categorized based on the optical density at 450 nm (OD₄₅₀) at the highest dilution tested (1/100) and 50% effective concentration (EC₅₀) values: + + + +, OD₄₅₀ > 3.0 and EC₅₀ > 5,000; + + +, OD₄₅₀ > 3.0 and EC₅₀ < 5,000; + +, 1.0 < OD₄₅₀ < 3.0; +, 0.2 < OD₄₅₀ < 1.0; -, OD₄₅₀ < 0.2. For the V3 glycan/V2 glycan, + + + indicates ≥50% effect on ID₅₀ and ID₈₀ of JRCSF.N332A/JRCSF.N160K, + + indicates ≥70% effect on ID₅₀ JRCSF.N332A/JRCSF.N160K, and + indicates ≥50% effect on ID₅₀ JRCSF.N332A/JRCSF.N160K. For MPER, + + + indicates ≥50% neutralization inhibited by MPER peptide competition (MPR.03) and ≥10-fold 7312.C1.Y720S/7312.Y720S reduction in neutralization, + + indicates ≥10-fold 7312.C1.Y720S/7312.Y720S reduction in neutralization, and + indicates ≥4-fold 7312.C1.Y720S/7312.Y720S reduction in neutralization.

MPER epitopes also was analyzed in samples from seven patients with cross-neutralizing profiles chronically infected for more than 365 dpi that were previously characterized with the same minipanel of viruses (Fig. 7).

Sera with antibodies directed against CD4bs were detected by ELISA in all groups of patients (Fig. 7 and 8 and N. Gonzalez, K. McKee, R. M. Lynch, I. S. Georgiev, L. Jimenez, E. Grau, E. Yuste, P. D. Kwong, J. R. Mascola, and J. Alcamí, submitted for publication). To determine whether virus neutralization was mediated by CD4bs antibodies contained in sera, RSC3 competition neutralization assays were carried out (Fig. 7 and 8B). None of the sera contained detectable CD4bs-directed neutralizing antibodies capable of being blocked by RSC3. Regarding the identification of V2 and V3 glycan-dependent HIV-1 neutralizing antibodies, a decrease in serum neutralization against JRCSF.N160K or JRCSF.N332A compared to the neutralization of wild-type JRCSF suggests the presence of neutralizing antibodies directed to glycans in V1V2 or V3, respectively (Fig. 7 and 9). V2 glycan-dependent HIV-1 NAb were abundantly found in serum samples from individuals infected with HIV for less than 365 dpi (7 out of 10). However, no antibodies against this region were detected in the samples from chronic individuals (0 out of 7), suggesting that V2 glycan epitopes are targeted predominantly by antibodies from early neutralizing responses in the studied patients. Nevertheless, V3 glycan-dependent antibodies were not detected in the samples

from patients with recent infection (<180 dpi), while antibodies against that epitope were detected in chronic patients (4 out of 7) (Fig. 7 and Gonzalez et al., submitted).

For the mapping of antibodies directed against the MPER of gp41 (Fig. 7 and 10A), sera were analyzed assessing neutralizing activity against 7312A and 7312A-C1 viruses, and 2 sera out of 6 from early chronic patients and 4 sera out of 7 from chronic patients appeared to contain MPER-reactive NAb. In 3 out of these 6 samples (serum DET 943 from an early chronic patient and sera 600-003 and 642-007 from chronically infected individuals), the specificity of the antibodies against MPER was inhibited with MPR.03 peptide, which covers the complete MPER domain (data not shown). Similar to what has been observed in V3, the MPER epitope was not targeted by antibodies in samples from patients infected for less than 180 dpi.

Overall, in samples from patients with recent infection (<180 dpi), cross-neutralization was mediated mainly by a single specificity among the known regions of susceptibility, whereas in most of the samples with early chronic infection (>180 dpi) and chronic infection (>365 dpi) it was associated with different specificities (Fig. 7).

DISCUSSION

It is now recognized that up to 50% of HIV-1 chronically infected subjects are able to generate cross-neutralizing antibody responses of significant breadth, and such responses are detectable in their plasma samples, on average, 2.5 years after HIV-1 infection (6, 50). However, on very rare occasions, cross-neutralizing antibodies have been detected close to the first year of infection (29, 30, 50). Still, it is currently unknown whether these responses can be detected even closer to the time of infection. In order to answer this question, here we screened for patients with cross-neutralizing responses within the first year of infection. Remarkably, we have identified 5 patients with a broad neutralizing response within the first 6 months of infection. In fact, one of these patients serum neutralized tier 1A, 1B, and 2 viruses from 3 different subtypes and 2 recombinant forms just 96 days postinfection. To our knowledge, this is the first time that bNA has been detected in such a relatively short time frame postinfection. Our data, together with the publications mentioned above showing bNA close to the first year of infection (29, 30, 50), serve as evidence that the human immune system, under certain circumstances, is capable of developing neutralizing responses against the HIV-1 envelope glycoprotein from a diverse group of HIV-1 isolates earlier than previously thought.

It is currently unknown whether the rare early generation of bNA is the result of a stochastic event or could be attributed to viral factors, host factors, or an interaction between both. For instance, patients capable of inducing broad neutralizing responses could have been infected with viruses with special envelope signatures that allow them to better engage bNA precursors, such as shorter V1-V4 envelope loops, specific signatures in the envelope CD4 coreceptor binding site, a shorter V1 envelope region, lower general envelope glycosylation, and a lower probability of glycosylation at the gp160 332 position (51–53).

Another viral factor that could contribute to the generation of an early bNA is viral diversity. The role of viral diversity driving bNA induction has been previously described in chronically infected patients (54–56). In the present study, we have observed in patients within the first year of HIV-1 infec-

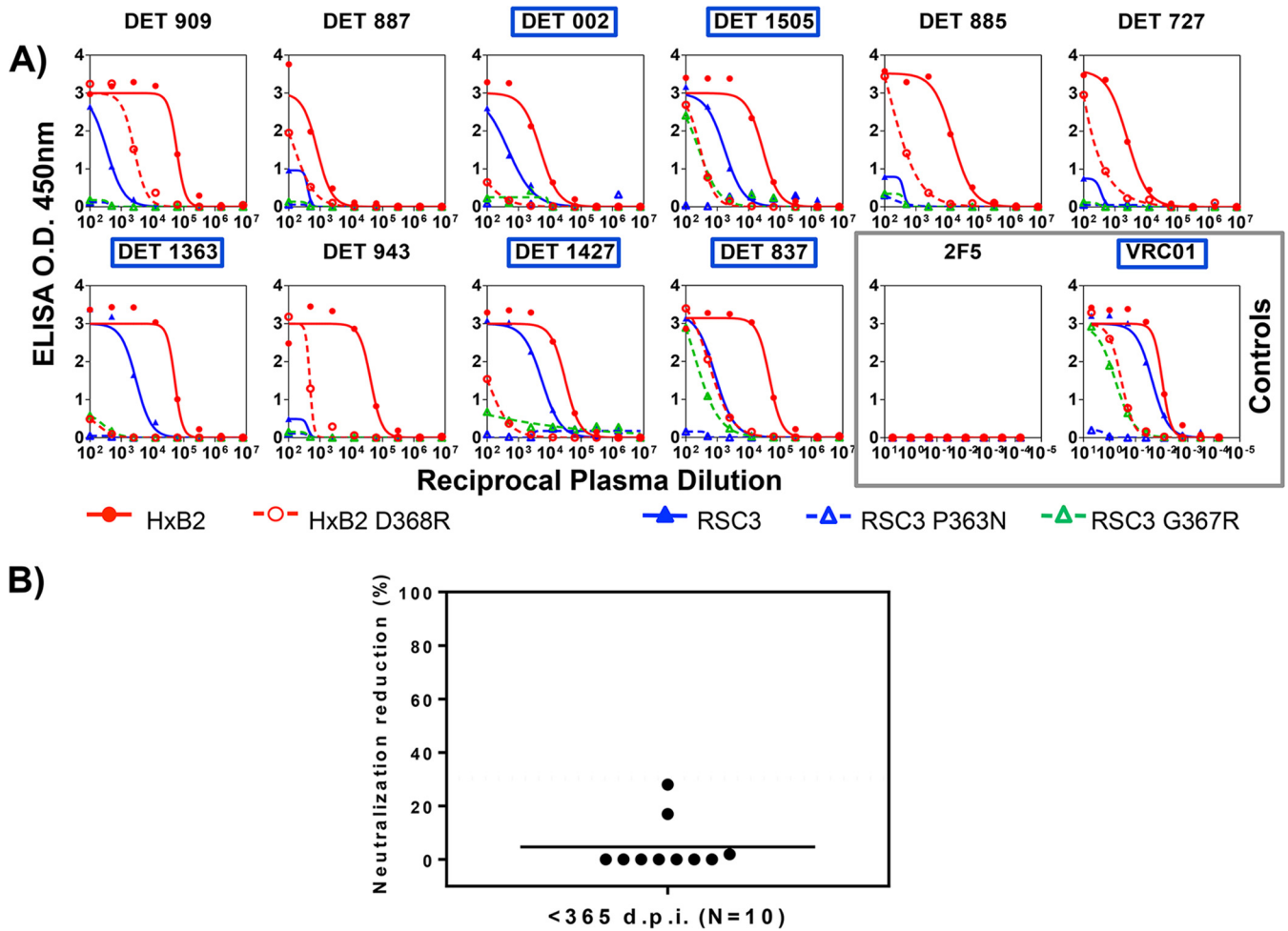


FIG 8 CD4 binding site neutralizing antibody specificity of the sera from patients with a broad neutralization profile within the first year of infection. (A) ELISA antigen binding profile of the sera from 10 patients capable of neutralizing at least one virus from 3 different subtypes from a previously described minipanel. Sera with antibodies against CD4bs were detected by ELISA with the following probes: HxB2 gp120wt, D368R, RSC3 containing the CD4bs, and the corresponding P363N and G367R mutants. Sera with reactivity to the CD4bs are indicated in blue. Monoclonal antibodies 2F5 and VRC01 have been used as controls and are indicated by a gray box. (B) Effect of resurfaced stabilized cores RSC3 and RSC3 P363N on the neutralization of RW020 by the selected sera. The net percent reduction in neutralization of RW020 attributed to RSC3 compared to RSC3 P363N is plotted (y axis). A reduction greater than 30% is considered positive.

tion a significant correlation between neutralization breadth and time postinfection. Considering that early in infection viral genetic diversity increases in an approximately linear fashion (57, 58), we hypothesize that one of the factors that contributed to an improved neutralization breadth in recent infection is the accumulated viral diversity.

In our study, we have identified five individuals who developed bNA within the first 6 months of infection (2 as early as 77 and 96 days postinfection). Unfortunately, the small number of patients of this type and limitations in sample availability that we have been able to identify makes the association of certain viral factors with the ability to induce bNA highly unlikely. Further studies with larger and more diverse patient cohorts would be necessary in order to determine the association between different viral factors, such as specific envelope signatures or the diversity of the viral quasispecies and the ability to induce broadly neutralizing responses in recent infection.

Among the host factors that could facilitate the induction of a

broader neutralizing response are certain characteristics of the immune status of the patient. In fact, there are several studies that show a correlation between certain B cell profiles and an improved ability to induce broad humoral immune responses against HIV in chronic patients. A study published in 2013 by our group showed an association between a broad neutralizing response and a partially restored B cell phenotype evidenced by increased frequency of naive B cells and a lower frequency of tissue-like and activated B cells (24). A recent report has also shown that an early preservation of CXCR5⁺ PD-1⁺ helper T cells and B cell activation was associated with induction of broadly neutralizing responses in chronic infection (59). These observations allow us to be more optimistic about the possibilities of the development of vaccination strategies based on the induction of bNAb, since they indicate that while this type of antibody is very difficult to induce in infected patients, they would be favored in healthy individuals with an intact B cell phenotype. In the present study, we found a similar effect in comparisons of the amplitude of the neutralizing

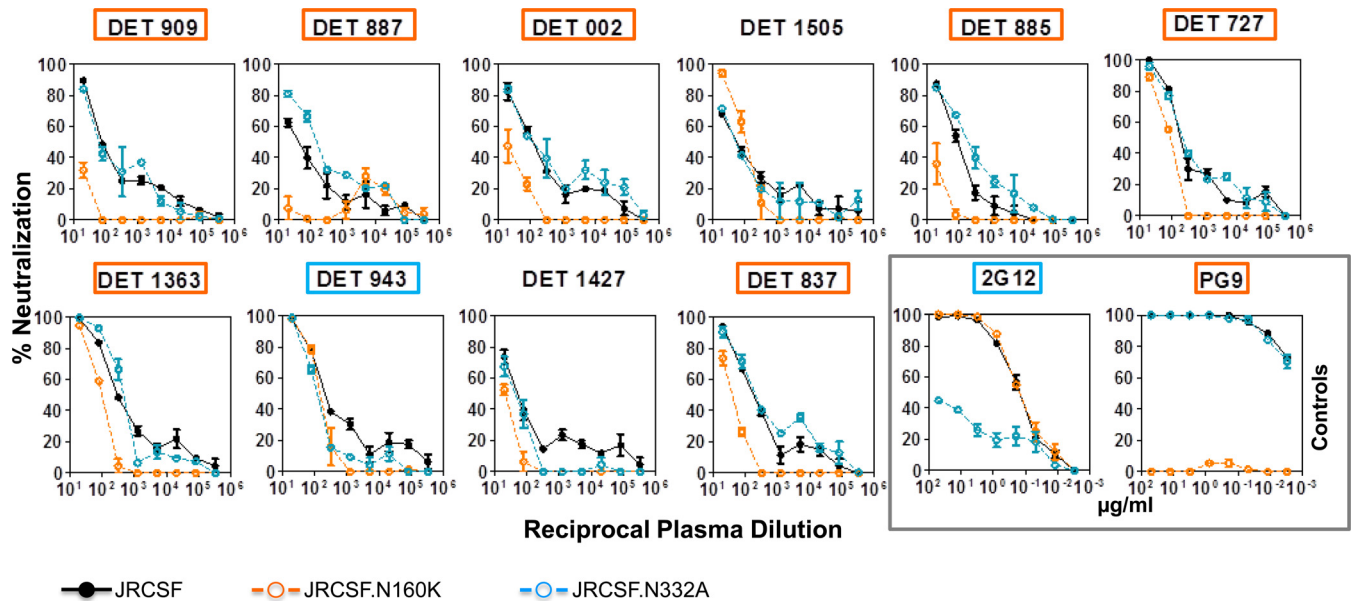


FIG 9 Detection of glycan-dependent HIV-1 neutralizing antibodies in sera from patients within the first year of HIV-1 infection. Sera with neutralizing antibodies against glycan-dependent motifs in the V2 and V3 loops are detected by neutralization assays with JRCSFwt and the corresponding N160K and N332A mutants (V2 and V3, respectively). Percent serum neutralizing activity sensitive to the indicated residue replacement was calculated using the equation $[1 - (IC_{50 \text{ variant}}/IC_{50 \text{ wild type}})] \times 100$. A sample is considered positive if there is a decrease in ID_{50} greater than or equal to 50% for the mutant relative to the wild-type virus. Reactivities to glycan-dependent motifs in V2 and V3 are indicated in orange and blue, respectively.

response in patients with healthy $CD4^+/CD8^+$ ratios (≥ 1) (47–49), within the first year of HIV infection, and with patients with lower ratios. Neutralization breadth was significantly higher in patients with $CD4^+/CD8^+$ ratios similar to those of healthy individuals. In contrast, these differences were not observed in chronically infected patients. Furthermore, several studies have suggested that symptomatic primary infection is associated with poorer subsequent outcomes and higher risk of disease progression (60–62). Consistent with this observation, we found that patients with an asymptomatic primary infection had significantly increased neutralization breadth that could be attributed to minor immune damage, also evidenced by higher $CD4^+/CD8^+$ ratios (Fig. 6).

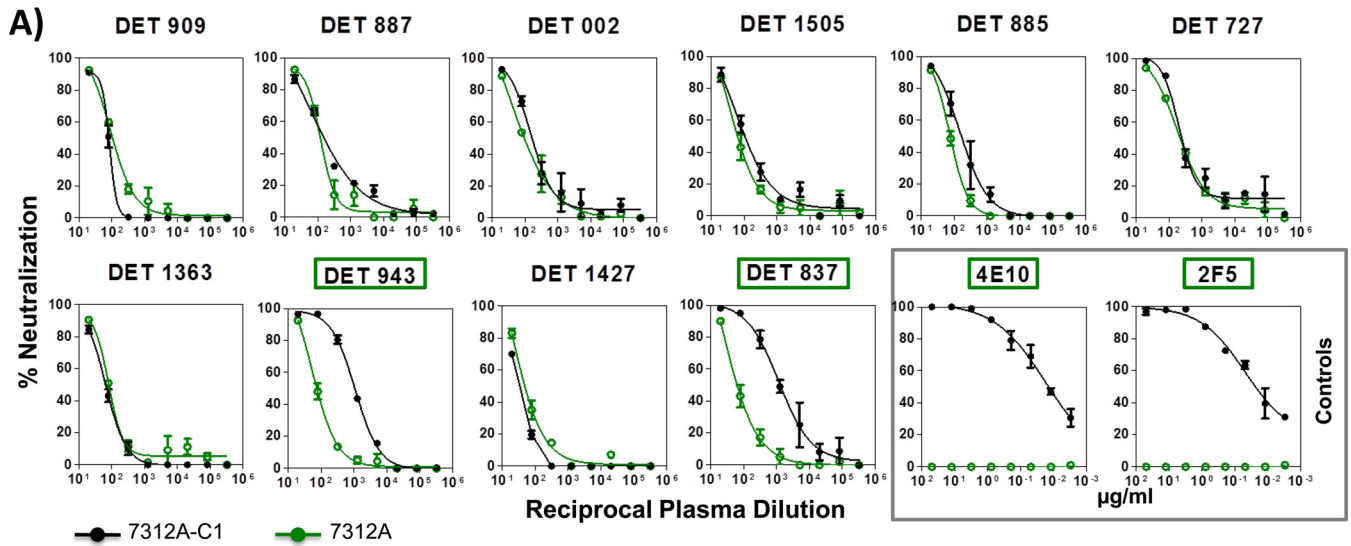
Another important aspect on which we have focused in the present study is the mapping of the epitopes recognized by patients who show improved neutralization breadth in recent infection. While it has been described that early cross-neutralizing antibody responses (on average, at 2.5 years after infection) targeted mainly epitopes within and around the CD4-binding site or epitopes present on the virion-associated trimeric envelope but not glycopeptide residues in the V1/V2 region (49), early development of broadly neutralizing antibodies in HIV-1-infected infants (at approximately 1 year after transmission from their mothers) has not been associated with any epitope specificity (63). Moreover, there is no information about epitope specificities of HIV-1-cross-neutralizing antibodies earlier than 1 year after infection.

In our study, we have observed that most of the cross-neutralizing responses during the first year of infection were highly associated with antibodies that recognize glycans in V2, while in chronic infection these responses were more related to the recognition of epitopes located on the CD4 binding site, in the glycan-dependent V3 site of vulnerability, and the MPER. In fact, no recognition of V2 glycans was found in patients with cross-neu-

tralizing responses in chronic infection when the same characterization was carried out (Gonzalez et al., submitted). However, although a significant trend is observed, these data are not statistically significant. A much larger screening, incorporating individuals with no bNA within the first year of infection, would be necessary to confirm this observation. RSC3-reactive antibodies identified by ELISA were not able to display neutralizing activity as confirmed by competition neutralization assays. In these samples the levels of antibodies directed against CD4bs may be too low to mediate neutralization; alternatively, the maturation of these antibodies could be insufficient to elicit strong avidity toward the CD4bs. Overall, in samples from patients with early recent and late recent infections (<180 dpi), cross-neutralization was mainly mediated by a single specificity (glycans in V2). In contrast, in most of the samples with early chronic (180 to 365 dpi) (Fig. 7) and chronic infections (>365 dpi; Gonzalez et al., submitted), it was associated with 1 to 3 different specificities (CD4 binding site, glycan-dependent V3 site of vulnerability single specificity, and the MPER) according to what has been previously described in chronically infected individuals (3, 46).

Our observation that cross-neutralizing antibodies induced within the first months after infection targeted mainly glycans in the V2 region is not surprising considering that neutralizing antibodies to the V1/V2 region are among the most prevalent cross-reactive neutralizing antibodies elicited by natural infection, and they can be elucidated relatively rapidly through initial selection of B cells with a long complementarity-determining region H3 segment and limited somatic hypermutation (3, 46, 55, 64–66).

The results of this study show that, contrary to what had been previously thought, the induction of neutralizing responses of significant breadth during the first 6 months of infection is feasible. We have also determined that this type of response is favored when the immune system of the patient has not undergone a ma-



B)

Serum	% Neutralization inhibited by peptide			
	MPR.Scr.02	MPR.03	2F5.01	4E10.22
DET 837	0	32	39	41
DET 885	0	39	46	19
DET 887	0	0	0	0
DET 943	0	71	60	52
DET 1363	0	41	0	41
DET 1505	0	0	4	21

FIG 10 Detection of antibodies specific for the membrane-proximal region in the sera from patients within the first year of HIV-1 infection. (A) Sera with neutralizing antibodies against the MPER are detected by neutralization assays with viruses 7312A-C1 and 7312A. Sera with reactivity to the MPER domain are indicated in green. (B) ID₅₀ titers in the presence of MPER-derived peptides. Selected sera were tested against 7312.C1 virus competing with soluble MPER peptides (MPR.Scr.02, MPR.03, 2F5.01, and 4E10.22). The peptide-specific neutralizing antibody response was calculated with the equation $[1 - (ID_{50} \text{ with peptide} / ID_{50} \text{ with mock peptide})] \times 100$. Values greater than 50% are shown in boldface.

for deterioration during the primary infection and is associated with the recognition of V1-V2 glycan-dependent motives. The study of the viral envelope proteins responsible for the induction of antibodies with improved neutralization breadth in recent infection, along with the characterization of bNAbs isolated from these patients, would provide valuable information for the design of an HIV-1 vaccine.

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